Supplementary Methods

qIF Analysis of phosphomarkers: A schematic illustration of the analysis of phosphomarkers in a DLBCL cell line is provided along with an identical illustration using screenshots from an actual analysis (Supplementary Figure S2). The original image is represented as a false two-color image by combining the fluorescent images of the DAPI nuclear stain (blue) and a phospho-specific marker (green). Six cells are illustrated, two with strong phospho-staining, two with weak phospho-staining, and two with no phospho-staining. The following steps are involved in the analysis:

1. The DAPI stain is used by Tissuequest™ to identify the nuclei based on image processing algorithms. From each nucleus the program uses additional algorithms to grow a boundary that effectively delineates all cells in the image. The resulting mask is illustrated by a thick green line around the nucleus and cell boundary. The resulting set of cell masks (termed "events") can be manually edited based on visual inspection. Manual editing and deletion of incorrect masks was used extensively in the TMA where artifacts (crinkling of the embedded specimen, overlaying of cells, blurred or out-of-focus tissue) can obfuscate image analysis.
2. Tissuequest then calculates the mean fluorescent intensity of the phospho-specific marker (shown in grey along with the cell mask to represent application of the mask to a single channel of the image). By choosing specific masks, one can measure intensities within different cellular compartments, such as the cytoplasm, or the total cell. Identified cells are displayed as scattergrams, indicating surface area/DNA and staining intensity. Just like in flow cytometry, each dot illustrates one individual cell.
3. For further analysis, cutoff parameters are based on staining tissues with fluorescently labeled, isotype-matched negative control antibodies. Gates are then set to define the positive staining

using the antibody of interest and the percentage of positive staining cells calculated. The software allows an individual point in the scattergram to be cross-referenced with visual inspection of the corresponding cell in the image and *vice versa* (forward-backward gating). Hence selection of appropriate gates can be confirmed visually for different specimens, for which the absolute fluorescent intensity may vary due to different degrees of background autofluorescence, staining with fluorophore, lamp intensity, etc.

Forward-backward gating was used to compare the use of total cell and cytoplasmic masks for the analysis of DLBCL phosphomarkers in cell lines and tissue specimens. Both methods gave comparable results for the percent positive cells. For DLBCL cell lines in which cells are morphologically homogenous and well dispersed, the use of a cytoplasmic mask is preferable since BCR phosphomarkers are almost exclusively cytoplasmic. In tissue specimens cells are morphologically heterogeneous, confluent and can vary dramatically between specimens in the relative proportion of cytoplasm to nucleus, which can give rise to artifacts. Hence the total cell mask was used for analysis of LYN and BTK phosphomarkers in the DLBCL TMA. For SYK however, a cytoplasmic mask is necessary to minimize the influence of nuclear staining by the (pY323) antibody.

Linear regression: the MLFR algorithm of Ripley & Thompson was applied to obtain a statistically unbiased estimate of the proportional change in %pBTK$^+$ vs. %pSYK$^+$ for the five Group I cell lines (Supplementary Table S1) using both untreated and crosslinked measurements (result of three independent experiments) weighted according to the relative error in both %pSYK$^+$ and %pBTK$^+$. The weighting function is $w = 1/(\lambda_i + \beta^2\kappa_i)$, where λ_i the standard error in y_i, κ_i the standard error in x_i, and β the value of the slope m. The FORTRAN77 program implementing numerical refinement of this algorithm uses the variance in place of the standard error. Due to the large range of variance in

%pSYK$^+$ and %pBTK$^+$ between untreated vs. crosslinked specimens (U2932 untreated %pSYK$^+$ has variance $s^2 = 8\times10^{-3}$ vs. HBL-1 crosslinked %pBTK$^+$ has variance $s^2 = 10^3$) a value of $s^2 + 1$ was used for numerical fitting. The validity of the assumption $\alpha = 0$ was confirmed by its refinement to a value of -0.8±0.8 for the cell pellet microarray and 2.0±0.9 for the TMA.

Significance: All *p* values correspond to the result of a 2-tailed heteroscedastic *t*-test. The determination of a critical value for ⟨pSYK,pBTK⟩ was based on critical values of the one-sided *t* distribution for the crosslinked specimens, i.e. $\langle \text{pSYK,pBTK} \rangle_{min,cell}(\text{BCR}^+) = \bar{x}_{\text{cr}} - t(\propto, \text{df}) \times s_{cr}$. A value of $\langle \text{pSYK,pBTK} \rangle_{\text{min}} = 15.1$ corresponds to to $\alpha = 0.05$ for the hypothesis 0<⟨pSYK,pBTK⟩<15 implies BCR$^-$, 15≤⟨pSYK,pBTK⟩<100 implies BCR$^+$. Since the variance of untreated specimens is much lower than for crosslinked specimens, this criteria is far more stringent against false positives than false negatives ($\alpha = 0.0025$ for untreated specimens with ⟨pSYK,pBTK⟩>15). Assuming the five cell lines analyzed represent a good model for DLBCL specimens within the TMA, this corresponds to a 5% chance of BCR$^+$ specimens being labeled as ⟨pSYK,pBTK⟩$^-$, but only 0.25% chance of a BCR$^-$ specimen being labeled as ⟨pSYK,pBTK⟩$^+$. The secondary criteria pLYN>15 corresponds to $\alpha = 0.01$ for crosslinked DLBCL Group I cell lines.

⟨pSYK,pBTK⟩ renormalization: Projection onto a line of best fit corresponds to a vector of length $d = (x_i + my_i)/\sqrt{1+m^2}$, hence normalization requires multiplication by $\sqrt{1+m^2}/(1+m)$. The slope of the regression line for %pBTK$^+$ vs. %pSYK$^+$ in the TMA is different than for the cell pellet microarray, m_{TMA}=0.895 (Figure 3, inset) vs. m_{cell}=0.47 (Figure 2B). Hence the corresponding normalized critical value of $\langle \text{pSYK,pBTK} \rangle_{\text{min}}(\text{BCR}^+)$=14.2 in the TMA is derived by the formula:

$$\langle \text{pSYK,pBTK} \rangle_{min,TMA}(\text{BCR}^+) = 15.1 \cdot \frac{1+0.47}{\sqrt{1+0.47^2}} \cdot \frac{\sqrt{1+0.895^2}}{1+0.895} = 14.2$$

qIF analysis of FOXO1: A schematic illustration of the analysis of FOXO1 localization in a DLBCL cell line is provided along with an identical illustration using screenshots from an actual analysis (Supplementary Figure S5). Cells are depicted as in Supplementary Figure S2, except that the second color now corresponds to FOXO1 (green), and cells also have varying fluorescent intensity within the nucleus as well as the cytoplasm. The following steps are involved in the analysis:

1. The DAPI stain is used to identify nuclei and grow cell boundaries as described above.
2. Analysis of the image by forwards-backwards gating and calculation of the percent positive cells is performed as before for both the total cell mask and the cytoplasmic mask. The scattergrams illustrates the point that the mean intensity of nuclear FOXO1 contributes to the mean intensity cell using of the total cell mask but are ignored in the cytoplasmic mask, hence cells with similar levels of cytoplasmic FOXO1 appear equivalent even if one has more FOXO1 in the nucleus and one has less. Images below the scattergrams show the cells counted as positive according to each analysis
3. The percent ratio of positive cells is used to estimate the number of cells that have significant FOXO1 cytoplasmic intensity.

Calculation of FOXO1 score: Assuming that FOXO1 nuclear intensity and FOXO1 cytoplasmic intensity are independent and normally distributed the value of the FOXO1 score, $F_{cyt}=100\times FOXO1_{cyt}^{+}/FOXO1_{tot}^{+}$, will be correlated with the average ratio of FOXO1 cytoplasmic and nuclear intensity per cell. Even if this assumption is not valid however the FOXO1 score will be correlated with increasing mean fluorescent intensity of cytoplasmic FOXO1.

Calculation of the FOXO1 score within the TMA is complicated by the fact that the number of $CD20^+$ cells varies between patient specimens. To account for this, the percent $FOXO1^+CD20^+$ must be divided by the percent $CD20^+$ for each core. Hence the formula for the FOXO1 score in the TMA is:

$$F_{\text{cyt}} = 100 \cdot \frac{[\text{FOXO1}^+\text{CD20}^+]_{\text{cyt}}}{[\text{FOXO1}^+\text{CD20}^+]_{\text{tot}}} \cdot \frac{[\text{CD20}^+]_{\text{tot}}}{[\text{CD20}^+]_{\text{cyt}}}$$

Due to a limitation™ of the Tissuequest software, the total cell mask must be generated independently for analysis of total cell and cytoplasmic intensity. This means that in principle the F_{cyt} score may produce a value greater than 100. In practice, this was only observed for a few cores in the TMA, all of which were strong positives, hence the F_{cyt} score was adjusted to 100.

Another possible source of error is that, because the scattergrams using total cell masks and cytoplasmic masks must be processed independently, there is no cross-referencing of positive events between the two analyses, which may mean that different events are being counted by the two methods. This error increases with decreasing values of $FOXO1^+CD20^+$, meaning that less confidence may be associated with low F_{cyt} values ($F_{\text{cyt}}<50$) than with high F_{cyt} values ($F_{\text{cyt}}>50$). For this reason specimens with $CD20^+<10\%$ of $FOXO1^+CD20^+<5\%$ were excluded from analysis. Visual assessment of actual specimens and correlation of the FOXO1 score with independent assessments by expert pathologists suggest that this error is not serious in practice.

Visual Scoring of FOXO1 localization by Experts

Visual scoring was performed by Expert 1 on the digitized images from IF staining of TMAs, or by Expert 2 on chromogenic (DAB) staining of TMAs (staining described in Methods section). Expert 1 did not score overall cell staining but made a subjective assessment of relative cytoplasmic localization (0 – nuclear, 1 – weak cytoplasmic, 2 – cytoplasmic). Expert 2 estimated stain intensity separately for nucleus and cytoplasm (0 – no staining, 1 - weak staining, 2 – strong staining, 3 – very strong staining). Correlation with F_{cyt} was assessed by box plots of F_{cyt} distribution for cases vs.

Expert 1's overall score and Expert 2's cytoplasmic score and calculation of polyserial correlation coefficient (`polycor` package, John Fox).

Image preparation of Qualitative Western Blots: Western blot images in this manuscript were obtained by the following procedure:

1. All samples were loaded on a single gel that was then probed with multiple antibodies.
2. The loading of each lane was adjusted to give qualitatively equal intensity of actin controls.
3. The dilution, incubation period and exposure time for each antibody was adjusted so that bands were visible for all cell lines and were sharp for weaker cell lines (Note: this may cause other lanes to appear overloaded or overexposed).
4. After scanning image levels were adjusted automatically in Adobe Photoshop to a common average (Note:variation in contrast may therefore lead films for different antibody to have slightly different backgounds).

Supplementary References

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